Detergent-Solubilized Bovine Cytochrome *c* Oxidase: Dimerization Depends on the Amphiphilic Environment†

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ABSTRACT: The extent to which bovine cytochrome *c* oxidase (COX) dimerizes in nondenaturing detergent environments was assessed by sedimentation velocity and equilibrium. In contrast to generally accepted opinion, the COX dimer is difficult to maintain and is the major oligomeric form only when COX is solubilized with a low concentration of dodecylmaltoside, i.e., ~1 mg/mg protein. The dimer form is intrinsically unstable and dissociates into monomers with increased detergent concentration, i.e., >5 mg/mg protein. The structure of the solubilizing detergent, however, greatly alters detergent effectiveness by inducing either monomerization or aggregation. Triton X-100 is most effective at solubilizing COX, but it destabilizes COX dimers, even at low concentration. Undecylmaltoside, decylmaltoside, and octaethyleneglycolmonododecyl ether ($C_{12}E_8$) are less effective at solubilizing COX. Each prevents COX aggregation at high detergent concentration, but also destabilizes the COX dimer. Other detergents, e.g., Tween 20, sodium cholate, sodium deoxycholate, CHAPS, or CHAPSO, are completely ineffective COX solubilizers and do not prevent aggregation even at 10−40 mg/mL. The transition from dimers to monomers depends on many factors other than detergent structure and concentration, e.g., protein concentration, phospholipid content and pH. We conclude that the intrinsic dimeric structure of COX can be maintained only after solubilization with low concentrations of dodecylmaltoside at near neutral pH, and even then precautions must be taken to prevent its dissociation into monomers.

Cytochrome *c* oxidase, in vivo, is generally assumed to be a dimeric complex with each monomeric unit containing 13 nonidentical subunits (*1*−*3*). Detergent extraction of the enzyme from the membrane, however, often disrupts the dimer. The nature of the solubilizing detergent, as well as the ionic strength and pH, have all been reported to affect self-association of purified, detergent-solubilized cytochrome *c* oxidase, but conditions favoring monomerization or dimerization have been controversial. Cytochrome *c* oxidase is clearly dimeric within two- or three-dimensional crystals (*2*, *4*, *5*). The solution structure of detergent solubilized cytochrome *c* oxidase is more ambiguous. Analytical ultracentrifugation has consistently shown that the enzyme is homogeneous and monomeric with $M_r \cong 200\,000$ after exposure to high pH (*6*), or with relatively high concentrations of detergent, e.g., Emasol 4130 (*7*), dodecylmaltoside (*8*), or Triton X-100 (*9*). Dimeric enzyme has been detected with low concentrations of Triton X-100 (*10*), but these preparations were not homogeneous and contained mixtures of monomers and dimers (*9*). Homogeneous and dimeric detergent-solubilized enzyme has never been clearly demonstrated by analytical ultracentrifugation.

The hydrodynamic size of the detergent-solubilized cytochrome *c* oxidase, as measured by gel filtration, is often interpreted as representing a dimeric complex (*11*−*14*). However, size measurements of detergent solubilized proteins are difficult to interpret in terms of protein mass due to the large contribution that bound detergent makes to the hydrodynamic size. For example, monomeric cytochrome *c* oxidase, solubilized by either dodecylmaltoside or Triton X-100, has a hydrodynamic radius that one would expect for a soluble protein with twice its molecular weight (*8*, *9*).

The dimeric structure of cytochrome *c* oxidase within the mitochondrial membrane and within two- or three-dimensional crystals is often interpreted as evidence that bovine cytochrome *c* oxidase is dimeric after solubilization by nondenaturing detergents. However, the wide variety of nonionic as well as ionic detergents that are commonly used to isolate and solublize the bovine enzyme complicates this situation even further, making it difficult or impossible to know the self-association status of cytochrome *c* oxidase during any experiment. We have, therefore, examined the hydrodynamic properties of bovine cytochrome *c* oxidase in a variety of detergents in order to clarify its self-association behavior in detergents that are commonly used in structural and functional studies on this enzyme. The results clearly indicate that homogeneous dimeric enzyme is very difficult, if not impossible, to obtain after detergent solubilization.

EXPERIMENTAL PROCEDURES

Materials. Cytochrome *c* type III (from Sigma) was used for enzyme activity determinations without further purifica-

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tion. Dodecylmaltoside ($C_{12}M$),[1] undecylmaltoside ($C_{11}M$), decylmaltoside ($C_{10}M$), CHAPS, and CHAPSO were purchased from Anatrace Inc. Ultrapure Triton X-100 was from Boehringer-Mannheim. Tween 20 was from Calbiochem, and $C_{12}E_8$ from Fluka. Sodium cholate was purchased from Sigma Chemical Co. and recrystallized from ethanol. Other chemicals were analytical grade.

Cytochrome c Oxidase. Keilin-Hartree particles were prepared from freshly frozen beef heart by the method of Yonetani (*15*). Cytochrome *c* oxidase was isolated by the modified method of Fowler et al. (*16*) as previously reported by Mahapatro and Robinson (*17*) with the addition of a second ammonium sulfate fractionation. After the final ammonium sulfate precipitation, the oxidase pellet was dissolved in 0.1 M NaH_2PO_4, pH 7.4, buffer containing 1.0% sodium cholate and 1.0 mM EDTA and stored at −70 °C at a concentration of approximately 30 mg/mL. The isolated complex contained 9.5−10 nmol of heme A/mg of protein [total heme A was measured using $\Delta\epsilon_{(606-630)} = 16.5\ mM^{-1}\ cm^{-1}$ for the fully reduced enzyme (*18*); protein concentration was measured by the Biuret method using bovine serum albumin as a standard]. The phospholipid content of enzyme preparations varied from 8 to 20 mol of P/COX monomer; lower values were usually obtained as the number of ammonium sulfate precipitations out of cholate solution was increased. Purified oxidase had a molecular enzyme activity of 340−350 μmol cytochrome *c* oxidized (μmol of cytochrome *c* oxidase)$^{-1}$ s^{-1} when assayed spectrophotometrically at 25 °C in 0.025 M, pH 7.0, phosphate buffer containing 1 mg/mL $C_{12}M$ (*19*, *20*). Enzyme isolated in Triton X-100 according to Soulimane and Buse (*21*) was a gift from Dr. M. Fabian in Dr. G. Palmer's laboratory at Rice University.

Analytical Ultracentrifugation. The molecular weight and sedimentation coefficient of COX solubilized in each detergent were determined by sedimentation velocity and sedimentation equilibrium experiments in a Beckman Optima XL-A Analytical Ultracentrifuge. Prior to analysis, sodium cholate in the stock enzyme was exchanged for the appropriate detergent, i.e., $C_{12}M$, $C_{11}M$, $C_{10}M$, Triton X-100, $C_{12}E_8$, Tween 20, CHAPS, or CHAPSO by the addition of 1.0−10 mg/mL of detergent (10−40 mg/mL for bile salts) to 1 mg/mL protein. Sodium cholate was removed by extensive dialysis vs two changes of buffer containing 0.1 mg/mL of the appropriate detergent (10−40 mg/mL for bile salts). By this procedure, sodium cholate was lowered to less than 0.03 mg/mL as quantified with [^{14}C]cholate. Sedimentation velocity experiments were done at 20 °C, at either 30 000 or 40 000 rpm, and absorbance scans were taken at either 8 or 12 min intervals at 422 nm (280 nm was used when the protein concentrations was less than 1 mg/mL). Sedimentation coefficients at fractional positions across the sedimentation boundary were calculated by the method described by van Holde and Weischet (*22*) to correct for the diffusion contribution. All data scans were obtained after the boundary cleared the meniscus, but before the plateau region began to be depleted (usually 12−20 scans). Sedimentation velocity data were also analyzed using the Stafford $g^*(s)_t$ time dependent derivative method (*23*) and the total area under each derivative curve normalized to unity. Time-dependent derivatives were generated from only six to eight scans starting at about 80 min with the 30 000 rpm data and at about 60 min with the 40 000 rpm data. The van Holde and Weischet analysis procedure is the method of choice for assessing sample homogeneity, a condition that would result in identical $s_{20,w}$ values at all fractional positions across the boundary. However, assessing the percent monomer and dimer within mixtures is more easily visualized by the Stafford $g^*(s)_t$ method. Both analyses were done using the UltraScan computer software developed by Borries Demeler. Use of this program has been described previously (*24*, *25*) and is available together with tutorial examples on the WWW at http://www.cauma.uthscsa.edu.

Sedimentation equilibrium measurements were performed at 10 °C and 6 000 rpm. Absorbance data at either 420 or 280 nm were collected when equilibrium was reached, generally after 24−36 h. The absorbance vs R^2 values were fitted to a model of two components, monomer and dimer, using the Marquardt−Levenberg algorithm in the Ultra-scan XLA program or SigmaPlot (Jandel Scientific) PC software and a 486DX microcomputer. The molecular weight of the monomer was assumed to be 205 000. The percentage of the two components was determined by integration of each component across the entire sedimentation cell. Integration was done using the four fitting parameters, i.e., the absorbance at the meniscus for monomer and dimer and the molecular weight of each. Two noninteracting components or two components in rapid or slow equilibrium cannot be distinguished by the above procedure. We have interpreted the sedimentation equilibrium data in terms of two noninteracting components, or a very slow equilibrium, based upon the following evidence: (1) the percent monomer and dimer evaluated by sedimentation velocity and equilibrium were in good agreement even though they were done at quite different protein concentrations; (2) mixtures of monomers and dimers yield two symmetrical Gausian distributions of $\partial c/\partial t$ and $\partial c/\partial r$ vs r in sedimentation velocity experiments; and (3) once dimers dissociate, the monomers do not reassociate to form dimers in any of the detergents (refer to Results and Discussion for details).

All molecular weight data were analyzed as suggested by Tanford et al. (*26*) to correct for the altered hydrodynamic size and effective partial specific volume due to bound detergents and phospholipids, i.e.,

$$M_{pr}(1 - \phi'\rho) = M_{pr}[(1 - \bar{\nu}_{pr}\rho) + \delta_{dct}(1 - \bar{\nu}_{dct}\rho) + \delta_{PL}(1 - \bar{\nu}_{PL}\rho)]$$

where M_{pr} is the molecular weight of the protein; ϕ' is the effective partial specific volume; $\bar{\nu}_{Pr}$, $\bar{\nu}_{dct}$, and $\bar{\nu}_{PL}$ are the partial specific volumes of protein, detergent, and phospholipid, respectively; δ_{dct} and δ_{PL} are the milligrams of bound detergent and phospholipid per milligram protein; and ρ is the solvent density.

[1] Abbreviations: COX, bovine heart cytochrome *c* oxidase; EC 1.9.3.1; $C_{12}M$, dodecylmaltoside; $C_{11}M$, undecylmaltoside; $C_{10}M$, decylmaltoside; $C_{12}E_8$, octaethyleneglycolmonododecyl ether; TX, Triton X-100; Tween 20, polyoxyethylene(20)sorbitan monolaurate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; MOPS buffer, 3-(*N*-morpholino)propanesulfonic acid titrated to the appropriate pH with NaOH; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography.

Table 1: Detergent Partial Specific Volumes and Binding Values

detergent	partial specific volume ($\bar{\nu}$, cm^3/g)	detergent binding (δ_{det}, g/g COX)
dodecylmaltoside ($C_{12}M$)	0.83[a]	0.35[b]
undecylmaltoside ($C_{11}M$)	0.83[c]	0.35[c]
decylmaltoside ($C_{10}M$)	0.83[c]	0.35[c]
Triton X-100	0.908[d]	0.54[e]
$C_{12}E_8$	0.973[f]	0.54[g]
Tween 20	0.869[f]	0.54[g]
sodium cholate	0.771[h]	0.16[i]
sodium deoxycholate	0.778[f]	0.165[j]

[a] Experimentally determined using an Anton Paar precision densimeter [value is very similar to that obtained by sedimentation equilibrium of $C_{12}M$ micelles in mixtures of H_2O and D_2O (*8*)]. [b] Evaluated from the excess detergent coeluting with COX from a DEAE Sephacel column [value is similar to that obtained by sedimentation equilibrium of $C_{12}M$-solubilized COX in mixtures of H_2O and D_2O (*8*)]. [c] Assumed to be the same as for $C_{12}M$. [d] Ref *26*. [e] Ref *9*. [f] Ref *35*. [g] Assumed to be the same as for Triton X-100. [h] Ref *36*. [i] Assumed to be the same as for sodium deoxycholate. [j] Ref *10*.

The contributions of bound phospholipids to $M_{pr}(1 - \varphi'\rho_0)$ were not considered in the calculation because their partial specific volumes are between 0.965 and 1.015 (*27*); therefore, the small amount of bound phospholipid contributes negligibly to the effective mass. Value for the partial specific volume of COX (cm^3/g) was $\bar{\nu}_{Pr} = 0.7428$ (calculated from the amino acid sequences of the 13 subunits). The partial specific volumes and detergent binding values that were used for each detergent are given in Table 1.

The effective radius (R_e) or Stokes's radius was evaluated from the sedimentation coefficient, protein molecular weight, the partial specific volumes of protein, detergent and phospholipid and reasonable values for the binding of detergent and phospholipid according to the equation

$$R_e = M_{pr}(1 - \varphi'\rho)/N6\pi\eta_{20,w}s_{20,w}$$

where $M_{pr}(1 - \varphi'\rho)$ is defined above and includes the protein molecular weight, all of the partial specific volumes and binding terms; N is Avogadro's number; and $\eta_{20,w}$ is the viscosity of water at 20 °C.

Subunit Composition. SDS−PAGE and Mono-Q FPLC ion-exchange chromatography combined with C_{18} reversed-phase HPLC (*28*) were used to confirm that all 13 COX subunits were present in both the monomeric and dimeric forms of enzyme.

RESULTS

Bovine heart cytochrome *c* oxidase (COX) was isolated from frozen heart tissue and purified in the presence of sodium deoxycholate and sodium cholate (refer to methods). The extent of COX dimerization was subsequently determined by analytical ultracentrifugation after exchanging a number of different nondenaturing detergents for the sodium cholate present in the isolated enzyme. In each detergent, COX exhibited different self-association behavior. With low concentrations of dodecylmaltoside, it was predominantly dimeric. In low concentrations of other detergents, however, it was either a mixture of monomers and dimers, or contained significant amounts of higher aggregates. In general, the percentage of dimers, monomers, or higher aggregates depends on many factors, including the detergent type, detergent concentration, solution pH, protein concentration and enzyme preparation. Specific details of the self-association behavior in each detergent are described in the following sections.

Effective Nonionic Detergents. (i) Dodecylmaltoside. Cytochrome *c* oxidase (1 mg/mL), solubilized with low amounts of $C_{12}M$ (~1 mg/mL), is predominantly dimeric with $s_{20,w}$ of 15.5−16 S, but always contains at least 15−20% monomeric enzyme with $s_{20,w}$ of 11.0−11.5 S (Figure 1A, open circles). Interpretation of this integral distribution plot in terms of only two sedimenting species is more easily understood when the same data are analyzed by the Stafford time-dependent derivative method (*23*). The time-dependent derivative, $g^*(s)_t$ (inset to Figure 1A), indicates only two populations of COX, monomers and dimers. The reasons for the incomplete dimerization in low concentrations of dodecylmaltoside are not known, but quantitative subunit analysis of our standard enzyme preparations indicates 15−20% of subunit VIb is often missing (*28*). Since subunit VIb participates in many of the contacts between the two monomers within crystalline dimeric COX (*5*), an incomplete complement of this subunit would explain the limitation upon dimerization in dodecylmaltoside. To prove this hypothesis, we would have to reassociate purified VIb with the 12 subunit COX complex. To date, we have not been able to accomplish this reconstitution, presumably due to interference of detergent with the reassociation process. Higher concentrations of dodecylmaltoside decrease the percentage of dimer, but do not cause further depletion of VIb, e.g., with 3 mg/mL $C_{12}M$ only 20−25% of COX is dimeric (Figure 1A, open squares). Our interpretation of the van Holde-Weischet integral distribution plots in terms of two species was verified by Stafford $g^*(s)_t$ analysis of the same data (inset Figure 1A, thick line). At a high $C_{12}M$ concentration (10 mg/mL), COX becomes 100% monomeric (Figure 1A, triangles). However, decreasing the $C_{12}M$ concentration to less than 1 mg/mL with 1 mg/mL protein does not increase the percentage of dimers, but generates significant amounts of higher aggregates (data not shown).

The absolute $C_{12}M$ concentration required for transition of dimers to monomers, or for solubilization of higher aggregates, varied with different preparations of enzyme. With a second preparation, for example, a significant proportion of COX remained aggregated at 1 mg/mL $C_{12}M$ (Figure 2, open circles, and inset, very thin line). Transition from higher aggregates to dimers and then to monomers each occurred at a higher $C_{12}M$ concentration (compare Figure 2 with Figure 1A). As was the case with the previous enzyme preparation, the maximum percentage of dimer was 80−85%. With other preparations of enzyme, only 15−20% was dimeric at 1 mg/mL $C_{12}M$, the remainder being monomeric (data not shown). The only significant difference we could detect between these preparations was the amount of bound phospholipid, which varied from a low of 6 to as much as 20 phospholipids/monomer. Preparations with higher phospholipid content consistently required higher concentrations of detergent to achieve the same ratio of monomers to dimers. The most likely reason for the different amounts of phospholipid was the number of ammonium sulfate precipitation steps that were done to purify the enzyme. Preparations in which purified enzyme was obtained in a single precipitation step had a high phospholipid content; those involving three or four precipitation steps during the enzyme purification

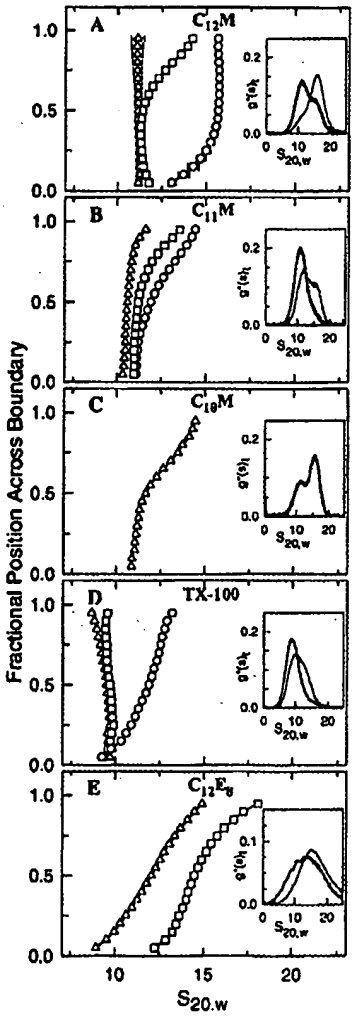

FIGURE 1: Dependence of COX sedimentation coefficients on concentration of nonionic detergents. The distribution of COX sedimentation coefficients was determined after solubilization of a single preparation of COX (8−10 mol of lipid-P/mol of monomeric COX) with either $C_{12}M$ (A), $C_{11}M$ (B), $C_{10}M$ (C), Triton X-100 (D), or $C_{12}E_8$ (E). Sedimentation data were analyzed by both the van Holde-Weischet method (main panels) and the Stafford $g^*(s)_t$ method (inset panels) as described in Experimental Procedures. In each experiment, purified cholate-solubilized enzyme was diluted to 1.0 mg/mL protein in 10 mM MOPS, pH 7.2 containing 1.0 mg/mL (○), 3.0 mg/mL (□), or 10 mg/mL (△), detergent. Cholate was removed by extensive dialysis against buffer containing 0.1 mg/mL of the appropriate detergent. Data in panel A represent averages from eight experiments; all other data are representative experiments. Only one or two sets of data are shown in the inset figures for clarity; the thin lines represent data obtained at lower detergent concentrations, the thick lines at higher detergent concentrations. These concentrations were: 1 and 3 mg/mL $C_{12}M$ (A), 1 and 10 mg/mL $C_{11}M$ (B), 10 mg/mL $C_{10}M$ (C), 1 and 3 mg/mL Triton X-100 (D), and 3 and 10 mg/mL $C_{12}C_8$ (E). In each experiment, COX concentration as a function of radial position was measured by absorbance at 422 nm. Data were collected at 10−12 min intervals during centrifugation of the sample at 30 000 rpm and 20 °C.

typically had a lower phospholipid content and a higher percentage of monomers.

Solution pH and protein concentration also affect the dissociation of COX dimers into monomers; higher pH or lower protein concentrations both induce monomerization. For example, with 1 mg/mL $C_{12}M$ and 1 mg/mL COX,

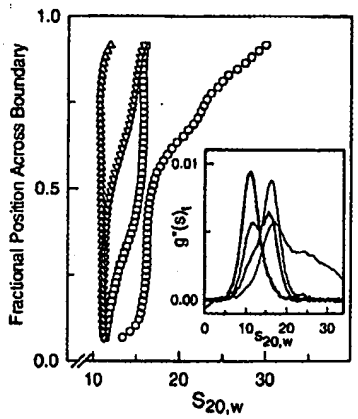

FIGURE 2: Dependence of COX sedimentation coefficients on $C_{12}M$ concentration for enzyme containing increased amounts of phospholipid. Distribution of COX sedimentation coefficients were determined after solubilization of a single COX preparation (18−20 mol of lipid-P/mol of monomeric COX) with either 1.0 mg/mL $C_{12}M$ (○), 3.0 mg/mL $C_{12}M$ (□), 5.0 mg/mL $C_{12}M$ (▽), or 10 mg/mL $C_{12}M$ (△). Data were analyzed by either the van Holde-Weischet method (main panel) or the Stafford $g^*(s)_t$ method (inset panel) as described in Experimental Procedures. In the inset, line thickness increases as the $C_{12}M$ concentration increases, i.e., the thinnest line is for data obtained with 1.0 mg/mL $C_{12}M$, the thickest line is for data with 10.0 mg/mL $C_{12}M$. Protein concentration across the sedimenting boundary was measured by absorbance at 422 nm as a function of radial position. Data were collected at 8 min intervals during centrifugation of the sample at 40 000 rpm and 20 °C.

increasing the pH from 7.2 to 8.8 converts predominantly dimeric enzyme into predominantly monomeric COX (Figure 3A). The pH effect is likely to be complex. As the pH is raised above 8, partial dissociation of subunits VIa, VIb, and III occurs (*28*, 29). Subunits VIa and VIb form the major contacts between the two monomers within a COX dimer (*5*); therefore, their removal would clearly favor monomer formation. However, this probably does not explain increased monomerization as the pH is raised from 7.2 to 8.0. Presumably the titration of amino acid side chains in this pH range increases the negative charge on each monomer which, in turn, destabilizes dimeric COX.

Induction of monomerization by decreasing the COX concentration is also somewhat complicated. If dimeric COX is diluted from 1 to 0.25 mg/mL while maintaining the $C_{12}M$ concentration at 1 mg/mL, COX becomes almost entirely monomeric (data not shown). In this case, dissociation of dimers is much greater than would be expected if dimers and monomers were in true equilibrium, but is entirely consistent with the detergent induced monomerization data of Figure 1A (the $C_{12}M$ to protein ratio increased to 4 during dilution). However, if dimeric COX is diluted with CMC amounts of $C_{12}M$, the data are more consistent with a very slow equilibrium between monomers and dimers with $K_d = (1.5-2) \times 10^{-6}$ M (Figure 3B). In the latter experiment, the ratio of excess detergent to protein remains constant; therefore, detergent induced monomerization is minimized.

These interpretations assume that sedimentation velocity data for $C_{12}M$-solubilized COX correspond to either a noninteracting, two-component model of monomers ($s_{20,w}$ = 11−11.5 S) and dimers ($s_{20,w}$ = 15.5−16 S), or a very slow equilibrium between monomers and dimers. The two component model was verified by sedimentation equilibrium after

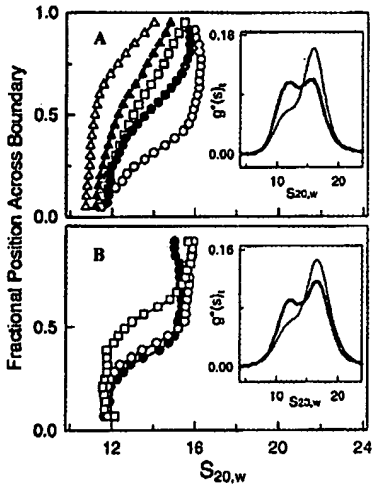

FIGURE 3: Effect of pH and protein concentration on sedimentation coefficients of $C_{12}M$-solubilized COX. (A) Increased pH favors COX monomerization. COX (8−10 mol of lipid-P/mol of monomeric COX) was solubilized with 1.0 mg/mL $C_{12}M$ in 20 mM MOPS buffer at pH 7.2 (○), pH 7.5 (●), or in 20 mM Tris-Cl buffer pH 8.0 (□), pH 8.4 (▲), and pH 8.8 (△). After extensive dialysis to remove cholate, self-association was determined by sedimentation velocity and analyzed by either the van Holde-Weischet method (main panel) or the Stafford $g^*(s)_i$ method (inset panel) as described in Experimental Procedures. Data were collected at 420 nm at 8 min intervals during centrifugation of the sample at 40 000 rpm and 20 °C. Inset, data obtained at pH 7.2 is shown as a thin line, data at pH 7.5 is shown as a thick line. (B) Lower protein concentration favors monomerization. COX was diluted to 1.0 mg/mL in 10 mM MOPS buffer, pH 7.2, containing 1.0 mg/mL $C_{12}M$. After dialysis to remove cholate, samples were diluted to 0.9 mg/mL (●), 0.6 mg/mL (○), or 0.3 mg/mL (□) with buffer containing CMC amounts of $C_{12}M$, i.e., 0.2 mM. Self-association of each sample was determined by either the van Holde-Weischet analysis (main panel) or the Stafford $g^*(s)_i$ analysis (inset panel) of sedimentation velocity data collected at 8 min intervals during centrifugation of the sample at 40 000 rpm and 20 °C. Protein concentration across the sedimenting boundary was measured by absorbance at 280 nm as a function of radial position.

correction for bound detergent. Weight average molecular weights of the monomeric and dimeric enzymes were evaluated by nonlinear least-squares fitting of the sedimentation equilibrium data to a two component model (205 000 Da monomer, 410 000 Da dimer). The percentage of each species was then calculated by integration across the entire sedimentation cell as described in Materials and Methods. These analyses support our interpretation of the sedimentation coefficients. With low $C_{12}M$-concentrations (1.0 mg/mL detergent and 0.1−1.5 mg/mL protein across the cell), the sedimentation equilibrium data fit a model consisting of 72% dimers and 28% monomers (Figure 4, curve a). At high $C_{12}M$-concentrations (10 mg/mL and 0.1−1.5 mg/mL protein across the cell), the data fit a model consisting of 95% monomers and 5% dimers (Figure 4, curve b).

(ii) Decyl- and Undecylmaltoside. Crystals used for determination of the three-dimensional, dimeric structure of COX, were grown using decylmaltoside ($C_{10}M$) solubilized enzyme (*4*, *5*). In this portion of our study, sedimentation velocity analysis was done on $C_{10}M$-solubilized COX to test whether this detergent is unique in its ability to stabilize the dimeric form. Decylmaltoside did not stabilize the dimer form of the enzyme and was considerably less effective than $C_{12}M$ in solubilizing COX, probably due to its relatively high

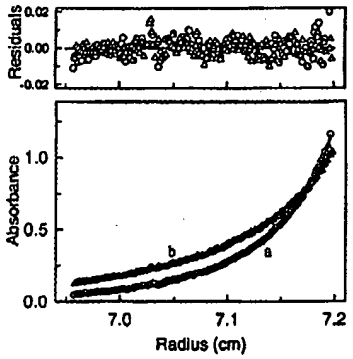

FIGURE 4: Sedimentation equilibrium analysis of $C_{12}M$-solubilized COX. Enzyme containing 8−10 mol of lipid-P/mol of monomeric COX was solubilized in 10 mM MOPS buffer, pH 7.2, containing either 1.0 mg of $C_{12}M$/mg of protein [line a (○)] or 9 mg of $C_{12}M$/mg protein [line b (△)]. Samples were prepared as described in the legend to Figure 1. Prior to sedimentation, the samples were diluted to 0.16 mg/mL protein. Absorbance vs the radial distance data were collected after centrifugation for 30 h at 6000 rpm at 10 °C. Solid lines are nonlinear least-squaresd fits to an ideal model of two noninteracting components, a monomer with M_{pr} = 205 000, a dimer with M_{pr} = 410 000, after taking into consideration detergent binding and the partial specific volumes of protein and detergent. Upper panel shows distribution of residuals, i.e., the difference between data and fitted line.

CMC (1.8 mM, i.e., 0.9 mg/mL) (*30*). With $C_{10}M$ concentrations far in excess of the CMC (5 mg/mL $C_{10}M$, 1 mg/mL protein), COX remained aggregated with a majority having an $s_{20,w}$ > 20 S (data not shown). At higher $C_{10}M$ concentrations (10 mg/mL), the enzyme was more dispersed, but still was a mixture of monomers and dimers, not completely dimeric (Figure 1C). We were unsuccessful in all attempts to prepare either monodisperse monomers or dimers in $C_{10}M$.

Solubilization of COX by $C_{11}M$ is more similar to $C_{12}M$ than to $C_{10}M$. Slight changes in the hydrocarbon chain length profoundly affect a detergent's ability to solubilize COX. At 1−3 mg/mL $C_{11}M$, COX is a mixture of 65−75% monomers and 25−35% dimers (Figure 1B, open circles and squares), values that are similar to those obtained for this preparation of enzyme after solubilization with similar amounts of $C_{12}M$. As was the case with $C_{12}M$, COX became a fairly homogeneous monomeric complex ($s_{20,w}$ = 10.5−11 S) with 10 mg/mL $C_{11}M$ (Figure 1B, open triangles; inset, thick line).

(iii) Triton X-100. Self-association of Triton X-100 solubilized COX is qualitatively similar to enzyme solubilized with $C_{12}M$, but the transition from dimeric to monomeric enzyme occurs at a lower detergent concentration. Therefore, the monomeric form of the enzyme is much more prevalent, and significant amounts of dimeric enzyme are difficult to obtain. In contrast to the $C_{12}M$ results, COX (1 mg/mL) solubilized with low amounts of Triton X-100, e.g., 1 mg/mL, contained at least 50% monomers ($s_{20,w}$ = 9.5−9.8 S), the remainder being dimeric ($s_{20,w}$ = 12−13 S) (Figure 1D, open circles; Inset thin line). As was the case with $C_{12}M$, the amount of dimer at these low Triton X-100 concentrations varied depending upon the phospholipid content of the enzyme preparation and was sometimes as low as 15%. Decreasing the Triton X-100 concentration to less than 1 mg/mL did not significantly increase the percentage of dimers, but induced only the formation of higher aggregates. Predominantly dimeric enzyme was never detected after

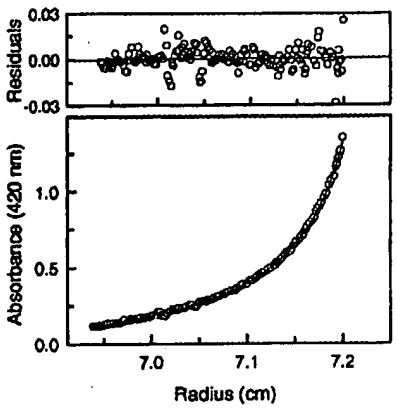

FIGURE 5: Sedimentation equilibrium analysis of $C_{12}E_8$-solubilized COX. Cholate-solubilized enzyme (0.16 mg/mL) containing 8−10 mol of lipid-P/mol of monomeric COX was solubilized in 10 mM MOPS buffer, pH 7.2, containing 1.6 mg/mL $C_{12}E_8$, i.e., 10 mg of $C_{12}E_8$/mg of protein and dialyzed to remove cholate. Absorbance vs the radial distance data were collected after centrifugation for 30 h at 6000 rpm at 10 °C. Experimental data (O) were fitted to an ideal model of two noninteracting components, a monomer with M_{pr} = 205 000, and a dimer with M_{pr} = 410 000 as described in Experimental Procedures. The partial specific volume of $C_{12}E_8$, is close to unity, therefore, detergent binding does not affect the hydrodynamic mass as much as does either Triton X-100 or $C_{12}M$. Upper panel shows distribution of residuals, i.e., the difference between data and fitted line.

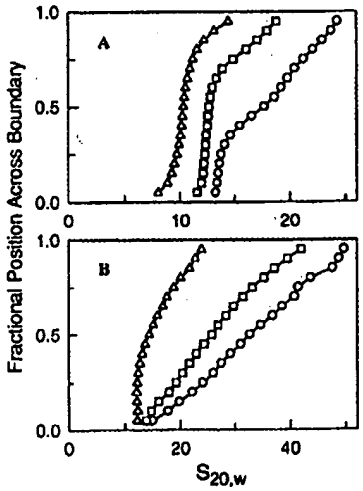

FIGURE 6: Sedimentation coefficients of COX solubilized with sodium cholate or Tween 20. All sedimentation data were analyzed by the van Holde-Weischet method after sedimentation at 30 000 rpm and 20 °C as described in Experimental Procedures. COX concentrations as a function of the radial position were measured at 10−12 min intervals using the absorbance at 422 nm. (A) Analysis of COX solubilized by sodium cholate. The distribution of sedimentation coefficients was determined after cholate-solubilized enzyme (9−10 mol of lipid-P/mol of monomeric COX) was diluted to 1 mg/mL protein in 20 mM Tris-Cl, pH 8.2 containing 10 mg/mL (O), 20 mg/mL (□), or 40 mg/mL (△) sodium cholate. Samples were dialyzed against buffer containing the appropriate concentration of cholate prior to analysis by sedimentation velocity. (B) Analysis of COX solubilized by Tween 20. The distribution of sedimentation coefficients was determined after the same enzyme preparation was diluted to 1.0 mg/mL protein in 10 mM MOPS, pH 7.2, containing 1.0 mg/mL (O), 3.0 mg/mL (□), or 10 mg/mL (△) Tween 20. Cholate was removed by dialysis against buffer containing 0.1 mg/mL Tween 20.

solubilization in Triton X-100. However, increasing the Triton X-100 concentration to more than 3 mg/mL consistently produced homogeneous monomeric enzyme (Figure 1D, open triangles and squares; inset, thick line). Consistent with the ease by which Triton X-100 dissociates COX dimers into monomers, enzyme preparations prepared in the presence of Triton X-100, i.e., by the method of Soulimane and Buse (*21*), were entirely monomeric. All of these data on Triton X-100 solubilized COX agree well with data previously collected with a model E analytical ultracentrifuge equipped with Schlierin optics (*9*).

(iv) Octaethyleneglycol-Monododecyl Ether. $C_{12}E_8$ was much less effective than Triton X-100, $C_{12}M$, or $C_{11}M$ in solubilizing COX. The enzyme tended to form high molecular weight species with $s_{20,w}$ = 15−30 S at the lowest $C_{12}E_8$ concentration (1 mg/mL, data not shown) and was still fairly heterogeneous at 3 mg/mL (Figure 1E, open squares; inset, thin line). Even with high concentrations of $C_{12}E_8$ (10 mg/mL), COX remained a mixture of monomers and dimers with sedimentation coefficients of 10 and 14 S, similar to its behavior in lower concentrations of the alkylmaltosides (Figure 1E, open triangles, inset, thick line). To test whether COX had become a mixture of monomers and dimers at 10 mg/mL $C_{12}E_8$, the molecular weight distribution was determined by sedimentation equilibrium. Equilibrium data could be fitted to a noninteracting two component model consisting of 70% monomers and 30% dimers (Figure 5), confirming that in the highest concentrations of $C_{12}E_8$, COX is solubilized to a mixture of monomers and dimers.

Ineffective Nonionic Detergents and Bile Salts. Sodium Cholate, Deoxycholate, CHAPS, CHAPSO, and Tween 20 Are Ineffective at Solubilizing Cytochrome c Oxidase. None of these detergents adequately solubilized COX to produce either COX monomers or dimers. Cholate-solubilized COX was a heterogeneous mixture of aggregated species with $s_{20,w}$ = 13−25 S, 12−20 S, and 8−15 S in 10, 20, and 40 mg/mL sodium cholate, respectively (Figure 6A). Deoxycholate, CHAPS, and CHAPSO gave results similar to cholate and all were ineffective at disrupting the COX higher aggregates even at high concentrations of detergent (data not shown). Tween 20, a detergent commonly used to solubilize COX during enzymatic assays, also did not produce a monomeric and/or dimeric complex (Figure 6B). In contrast to the other nonionic detergents studied, COX remained a heterogeneous mixture ($s_{20,w}$ = 10−22 S) even at 10 mg/mL Tween 20.

Apparent Irreversibility of Detergent-Induced Monomerization. The sedimentation velocity and equilibrium data indicated that the conversion of dimeric to monomeric COX was irreversible or in very slow equilibrium. All data could be fitted by assuming two noninteracting species. Detergent binding to monomers probably explains the apparent irreversibility. Once a dimer dissociates into two monomers, detergent would coat the newly exposed apolar surfaces and prevent any subsequent protein−protein contact. The only way that two monomers could then interact would be if bound detergent dissociated from these surfaces. This would not occur unless the free detergent concentration was lower than the CMC. The data obtained at different COX concentrations support this hypothesis. When COX was diluted with 2 mM $C_{12}M$ (1 mg/mL), conditions that increase the detergent-to-protein ratio, dimeric COX irreversibly dissoci-

ated into monomers. However, the dilution of dimeric COX with buffer containing CMC amounts of $C_{12}M$ produced results consistent with a slow equilibrium between dimeric and monomeric COX (refer to Figure 3B). Several attempts were made to decrease the solubilizing detergent concentration in order to induce reformation of dimers. In one case, Triton X-100-induced monomers were prepared, and the excess detergent was removed by either DEAE ion-exchange chromatography, adsorption of the detergent on Bio-Beads SM-2, membrane ultrafiltration, or exchange of $C_{12}M$ for bound Triton X-100. In a second case, $C_{12}M$-induced monomers were prepared, and excess detergent was removed by ion-exchange chromatography. In each instance the final detergent concentration was successfully lowered, or exchanged, but specific self-association was not detected by sedimentation velocity. Enzyme treated in these ways either remained monomeric or nonspecifically associated to form large heterogeneous aggregates.

Functional Consequences of Detergent-Induced Monomerization. Monomeric COX is functionally competent in terms of its electron transport activity, cytochrome *c* binding, and ligand binding. It is experimentally impossible to compare the electron transport activity of COX monomers and dimers since COX dimers dissociate into monomers when they are diluted into the assay buffer that typically contains 0.5−1 mg/mL detergent. Therefore, all enzymatic assays of detergent-solubilized COX are measures of the activity of COX monomers. Nevertheless, monomeric COX exhibits normal biphasic cytochrome *c* kinetics when assayed polarographically (*31*), has normal enzyme activity when assayed spectrophotometrically (*31*), has normal high and low affinity cytochrome *c* binding sites (*32*), and has normal biphasic cyanide binding kinetics (*33*). However, the proton-pumping activity of COX is probably dependent upon a dimeric complex (*34*). We were not able to compare the proton pumping activity of dimeric COX with detergent-solubilized monomeric complexes since the latter spontaneously dimerized when reconstituted into phospholipid vesicles (A.M. and N.C.R., unpublished results).

DISCUSSION

The sedimentation studies reported here indicate that detergent-solubilized COX can be dimeric after solubilization by detergents, but only if solubilized with low concentrations of certain alkylmaltosides. However, solutions of detergent-solubilized dimers always contained at least some monomers and/or higher aggregates. The highest percentage of dimers achieved was 80−90% after solubilization in low concentrations of dodecylmaltoside. In all other detergents and higher concentrations of the dodecylmaltoside, the percentage of dimers is always much less and can be zero. From this study, we can make several general conclusions: (1) COX dimerization is highly dependent upon the type of solubilizing detergent; (2) detergent induced dissociation of dimers to monomers is irreversible in nonionic detergents; and (3) the sedimentation coefficients of COX monomers or dimers is highly dependent upon the solubilizing detergent. The basis for each of these conclusions is discussed in the following sections.

Dimerization of COX in Different Detergents. The self-association behavior of detergent-solubilized COX is strongly dependent upon both the type and concentration of detergent. The solubilized enzyme can be predominately dimeric, but is often a mixture of monomers and dimers, monomeric, and/or highly aggregated. Some detergents, e.g., Triton X-100, dodecylmaltoside ($C_{12}M$), or undecylmaltoside ($C_{11}M$), are effective in minimizing the amount of higher aggregates, but they also induce the dissociation of the dimers into monomers as the detergent concentration is increased. The percentage of dimers in these detergents is difficult to predict and must be experimentally determined for each enzyme preparation. It is strongly dependent upon many factors, including phospholipid content, solution pH, type of detergent, and concentrations of detergent and protein. Only by carefully titrating the enzyme with dodecylmaltoside were we able to maximize the dimeric form of COX and obtain predominately dimeric enzyme.

Two other detergents, Triton X-100 and undecylmaltoside ($C_{11}M$), are also effective solubilizing detergents, but COX dimers are less stable in these two detergents and thus high percentages of dimer are difficult to obtain. However, $C_{12}E_8$ and decylmaltoside ($C_{10}M$) are less effective solubilizing agents than dodecylmaltoside, Triton X-100, or undecylmaltoside, but each begins to induce dissociation of dimers at the high concentrations required for complete solubilization. The fact that decylmaltoside does not disrupt COX aggregates and it stabilizes protein−protein contacts may explain why it was the detergent of choice for crystallizing COX for structure determination (*5*). A fourth group of detergents, e.g., sodium cholate, deoxycholate, CHAPS, CHAPSO, or Tween 20, is completely ineffective at preventing COX aggregation and cannot be used to completely solublize the enzyme. Upon the basis of results obtained with each detergent, we can summarize their effectiveness at solubilizing COX to be Triton X-100 > $C_{12}M \cong C_{11}M$ > $C_{10}M \cong C_{12}E_8$ > Tween 20 ≅ cholate ≅ deoxy-cholate ≅ CHAPS ≅ CHAPSO.

Apparent Irreversibility of Detergent-Induced Monomerization in Nonionic Detergents. COX monomers and dimers almost always behave as noninteracting species in each of the nonionic detergents used in this study. This behavior could be due to true nonequilibrium or to a very slow equilibrium between monomers and dimers. The enzyme dilution results, in which COX was diluted with CMC amounts of $C_{12}M$ (Figure 3B), suggest that the two COX forms are in true equilibrium; however, the process must be very slow since all other data can be fitted assuming two noninteracting species. Furthermore, we could obtain no experimental evidence that monomerization is reversible in any of these single detergent environments. Irreversibility of the monomerization step would seem to be thermodynamically improbable since monomer−dimer protein systems are usually reversible. However, the presence of solubilizing detergent perturbs the equilibrium and makes this system different. Once a dimer dissociates into two monomers, detergent immediately coats the dimer interface of each monomer and prevents monomer reassociation. The only way that the two protein surfaces could interact to form a dimer is if the surface were free of detergent which is highly unlikely at detergent concentrations 10−100 times the critical

micelle concentration. In other words, high detergent concentrations push the reaction toward monomers. Detergent binding thermodynamically traps COX in the monomeric form, thereby, preventing reversibility of the reaction.

Dilution of COX with CMC levels of detergent eliminates detergent-induced dissociation of dimers and produces results that are consistent with a true equilibrium situation. However, reversal of this process is still problematic since it would require (1) concentrating the protein without concentrating the detergent, and (2) overcoming the very slow kinetics of reassociation. In the present study, we attempted to reverse the dissociation process by removing excess detergent, but as is often the case with membrane proteins in the presence of low concentrations of detergents, these procedures produced only aggregated enzyme, not a dimeric product. Reformation of dimers from detergent-solubilized monomers is not inherently impossible, but is prevented by the large polar groups of the bound detergent.

Sedimentation Coefficient of Monomeric and Dimeric COX Depends on the Solubilizing Detergent. Care must be taken in assuming that monomeric or dimeric COX has a particular sedimentation coefficient unless values are compared using the same solubilizing detergent. For example, the sedimentation coefficient for a Triton X-100 solubilized dimer ($s_{20,w}$ = 12−13) is similar to that of a $C_{12}M$-solubilized monomer ($s_{20,w}$ = 11−12), but significantly smaller than a $C_{12}M$-solublized dimer ($s_{20,w}$ = 15−16). Two factors cause this difference. (1) The polar headgroup of Triton X-100 is much larger than that of $C_{12}M$, causing the COX-TX complex to have a larger hydrodynamic radius and a smaller sedimentation coefficient than the COX-$C_{12}M$ complex. (2) The partial specific volume of Triton X-100 (0.908 cm^3/g) is larger than that for $C_{12}M$ (0.83 cm^3/g). Therefore, bound Triton X-100 increases the hydrodynamic mass less than bound $C_{12}M$; however, this is largely compensated by the larger amount of bound Triton X-100 (0.54 g TX/g protein vs 0.35 g $C_{12}M$/g protein). The larger hydrodynamic radius of the COX-TX complex is the most significant reason that the sedimentation coefficients are so different in the two detergents. The hydrodynamic radii of monomeric and dimeric COX in each detergent were evaluated from the sedimentation coefficients and found to be (a) 62−64 and 80−84 Å for COX monomers and dimers in Triton X-100 and (b) 49−51 and 71−74 Å for COX monomers and dimers in $C_{12}M$. The large effect of the detergent's polar headgroup size upon the hydrodynamic radius of the resulting detergent−COX complex may explain some of the discrepancies in the literature when size exclusion chromatography has been used to measure the presence or absence of COX dimeric forms.

Conclusions. We conclude that detergents can only partially mimic the native hydrophobic environment supplied by the phospholipid bilayer in vivo. Most nonionic detergents can maintain the native structure of monomeric COX, but many detergents disrupt the native dimeric enzyme. Only under certain carefully controlled experimental conditions with either dodecyl- or undecylmaltoside were we able to maintain the dimeric COX structure. The only way that one can be confident that a preparation of detergent-solublized COX is dimeric is if its presence is verified by a reliable biophysical method.

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